

From the Institute of Physiology, University of Lund, Sweden

CHOLINE ACETYLTRANSFERASE IN  
SALIVARY GLANDS AND SOME OTHER  
ORGANS, AND ITS DEPENDENCE ON  
NERVOUS ACTIVITY

By

Jörgen Ekström

LUND 1975





# CHOLINE ACETYLTRANSFERASE IN SALIVARY GLANDS AND SOME OTHER ORGANS, AND ITS DEPENDENCE ON NERVOUS ACTIVITY

av

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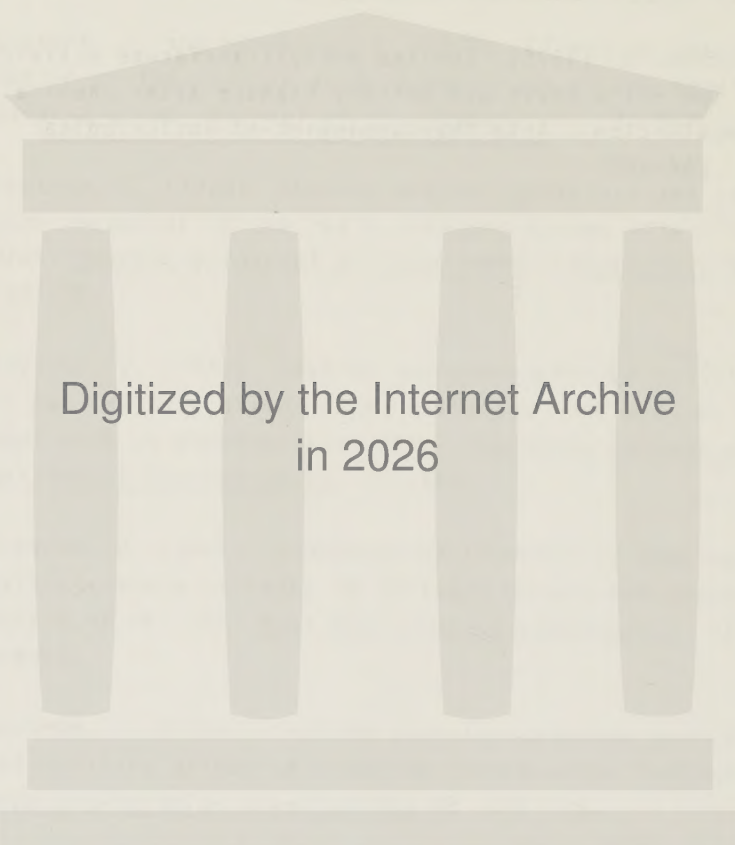
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This thesis is based on the following papers which in the text will be referred to by their Arabic numerals:

1. Ekström, J. and Holmberg, J. (1972). Choline acetyltransferase in the normal and parasympathetically denervated parotid gland of the dog. Acta physiologica scandinavica 86. 353-358.
2. Ekström, J. and Holmberg, J. (1972). Effect of decentralization on the choline acetyltransferase of the canine parotid gland. Journal of Physiology 222. 93-94P.
3. Ekström, J. (1973). Choline acetyltransferase and secretory responses of the rat's salivary glands after liquid diet. Quarterly Journal of Experimental Physiology 58. 171-179.
4. Ekström, J. (1974). Choline acetyltransferase activity in rat salivary glands after cellulose rich diet or treatment with an atropine-like drug. Quarterly Journal of Experimental Physiology 59. 191-199.
5. Ekström, J. (1975). Compensatory increase in choline acetyltransferase activity in salivary glands and diaphragm muscle of the rat. Acta physiologica scandinavica (In the press).
6. Ekström, J. (1974). Choline acetyltransferase activity in rat salivary glands enlarged by isoprenaline treatment. Acta physiologica scandinavica 92. 272-275.
7. Ekström, J. (1970). Distribution of choline acetyltransferase in the hearts of mammals. Acta physiologica scandinavica 80. 73-78.



8. Ekström, J. (1974). Choline acetyltransferase in the heart and salivary glands of the rat after physical training. Quarterly Journal of Experimental Physiology 59. 73-80.
9. Ekström, J. (1972). Choline acetyltransferase in salivary glands after surgical and chemical sympathectomy. Acta physiologica scandinavica 86. 539-545.
10. Ekström, J. (1975). Choline acetyltransferase activity in the rat's heart and urinary bladder after chemical sympathectomy. Acta Pharmacologica et Toxicologica 36. 284-288.



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## INTRODUCTION

In mammals synthesis of acetylcholine is in general confined to nervous structures such as the central nervous system, autonomic preganglionic nerves, parasympathetic postganglionic nerves and somatomotor nerves (see Feldberg and Vogt, 1948; Hebb, 1957, 1963; Nordenfelt, 1965 a; Fonnum, 1973). The placenta of higher primates is the unambiguous exception, since acetylcholine is formed here, although there is a complete lack of nerves in the tissue (Comline, 1946; Hebb and Ratković, 1962). The role of acetylcholine in the placenta is not well understood. The general view of the function of acetylcholine in nerves is that of a neurotransmitter. The ability to form acetylcholine is primarily dependent on the presence of choline acetyltransferase, the enzyme that transfers the acetyl group from acetyl-Coenzyme A to choline (EC.2.3.1.6). In nervous tissue choline acetyltransferase is thought to be manufactured by the nerve cell body and moved along the axon mostly at a slow rate with the bulk of axoplasm to the nerve endings (Hebb and Waites, 1956; Hebb and Silver, 1961; Hebb, 1962; Frizell, Hasselgren and Sjöstrand, 1970; Ekström and Emmelin, 1971; Fonnum, Frizell and Sjöstrand, 1973; Tuček, 1975). In this region, where the main synthesis of acetylcholine is supposed to take place (see Hebb, 1972), the enzyme accumulates (Hebb, Krnjević and Silver, 1964; Fonnum, Frizell and Sjöstrand, 1973).

In salivary glands the effects of various kinds of denervation procedures on the choline acetyltransferase activity have been studied extensively by Nordenfelt (see Thesis, 1965a).

In the cat and the rabbit parotid glands a profound fall in the enzyme activity was observed following parasympathetic denervation, caused by severance of the auriculo-temporal nerve; the fall was rapid, reaching its lowest value already on the 3rd day, as shown in the rabbit parotid gland. These findings most likely indicate a neuronal localization of the enzyme in the glands. However, some enzyme activity did remain, suggesting either the presence of unsevered cholinergic neurones or sources for the enzyme outside the neurones; it was not further reduced by degeneration of the sympathetic fibres. In this connexion the puzzling observation was made that sympathectomy could, in fact, increase the choline acetyltransferase activity in some salivary glands, provided the parasympathetic pathway was intact (Nordenfelt, 1964 a, 1965b).

When preganglionic instead of postganglionic parasympathetic fibres were cut, Nordenfelt (1963, 1964 b) discovered that this was also followed by a fall in the choline acetyltransferase activity; the reduction was not as marked as that seen after postganglionic denervation. In the submaxillary gland of the rabbit and the cat the fall could at least partly be explained by degeneration of preganglionic nerve fibres within the glandular parenchyma. However, in the cat parotid gland the synapses between pre- and postganglionic neurones are located outside the gland, and since a decrease in the enzyme activity on parasympathetic decentralization was noticed in this gland also, some other explanation had to be sought for. Nordenfelt (1964 b) put forward the hypothesis that abolishment of impulse traffic in the postganglionic neurone by disconnecting it from the central nervous system



was responsible for the fall in the enzyme activity in the gland.

This thesis describes experiments on the choline acetyltransferase activity under various conditions particularly in salivary glands, but also in some other organs (heart, diaphragm, urinary bladder). The first problem dealt with (section A in Results and Discussion) is whether the choline acetyltransferase still found in parotid glands after degeneration of the postganglionic parasympathetic fibres in the auriculo-temporal nerve can be removed by section of some other nerves. It has long been suspected that parasympathetic fibres reach the parotid gland by other routes also (see Babkin, 1950; Emmelin, 1967; Emmelin and Holmberg, 1967), and such nerves were recently detected in the parotid gland by Holmberg (1971) along the internal maxillary artery and, to some extent, in the facial nerve. By cutting these nerves and the auriculo-temporal nerve the choline acetyltransferase activity of the parotid gland could be lowered under the limit of detection, suggesting that the enzyme is contained in nerves.

Next, the effect of parasympathetic decentralization on the choline acetyltransferase activity of the dog parotid gland was investigated (section B). In the cat this operation was found to damage some sympathetic postganglionic fibres, introducing a possible source of error (Nordenfelt, 1964 b). This is not the case in the dog (Garrett and Holmberg, 1972). In experiments on dogs it was confirmed that parasympathetic decentralization reduces the activity of the enzyme in the parotid gland.

In the following eight sections (C-J) various procedures were used which aimed at changing the impulse traffic in cholinergic nerves. Most of these experiments were made on salivary glands of rats. It was thought that a liquid diet, lowering the demands on the salivary glands, might reduce the flow of impulses in the main secretory nerves of these glands, the parasympathetic (C). Conversely, food with a high content of cellulose might have the opposite effect (D). In rats the salivary glands take part in thermoregulation, and in connexion with exercise the salivary glands are activated and saliva is spread on the body surface to evaporate (E). Dryness of the oral and pharyngeal mucosa will reflexly stimulate the salivary glands; the dryness can be brought about by atropinization (F) or by ligation of most of the salivary ducts (G). The outcome of these various experiments was to support generally the hypothesis that increased impulse flow raises, and decreased flow lowers the choline acetyltransferase activity in salivary glands. Since procedures stimulating for a long time salivation are known to increase the weight of salivary glands (see Ohlin, 1966) it was thought desirable to study whether a gland enlargement in itself would affect the enzyme activity; a very effective way of increasing the size of salivary glands in rats is to administer isoprenaline repeatedly (H). Attempts were also made to increase the impulse flow in the cardiac vagus fibres (I) and in the cholinergic, somatomotor fibres of the phrenic nerve (J).

Finally the effect of sympathectomy on the activity of choline acetyltransferase was studied in salivary glands, and also in hearts and urinary bladders (K). The original experi-

ments (Nordenfelt, 1964 a, 1965 b) showed that sympathetic denervation increases the enzyme activity in some salivary glands but not in others. In the present experiments the conditions under which an increase occurs were investigated. It could be hypothesized that in salivary glands loss of the sympathetic secretory pathway might increase the reflexly induced impulse flow in the parasympathetic nerves, thus causing a rise in the enzyme activity, and some arguments were found in support of such a view. However, loss of secretory impulses in the sympathetic nerves does not seem to provide the whole, or even the main explanation of the phenomenon.

The experimental animals were adult cats, dogs, golden hamsters, guinea-pigs, mice and rats. The rats were of a Sprague-Dawley strain bred at this Institute.

### Surgical procedures

Denervation of salivary glands. These operations were carried out in ether anaesthesia, in the dog after induction with sodium thiopentone.

Parasympathetic postganglionic denervation of the parotid gland in dogs (1) and rats (4) was done by severing the auriculo-temporal nerve, exposed essentially as described by Burgen (1964), where it emerges from the base of the skull. Additionally, in dogs the adventitia of the internal maxillary artery was stripped in order to damage nervous strands on the vessel (Holmberg, 1971) and the facial nerve was cut just outside the stylomastoid foramen (1). Parasympathetic preganglionic denervation of the parotid gland in dogs was done by dividing the tympanic nerve in the middle ear (2). Sympathetic postganglionic denervation was done by excision of the superior cervical ganglion (4, 9).

Denervation of the hemidiaphragm muscle. This operation was made on rats in pentobarbitone anaesthesia. To suppress bronchial secretion atropine sulphate was given. Artificial respiration was given by a pump through a fine glass tube which was inserted into the trachea through a small cut. The intrathoracic approach to the phrenic nerve was chosen, since some of the cervical branches forming the nerve might escape

section if done in the neck region (Sola and Martin, 1953; Emmelin and Malm, 1965). The intact half of the diaphragm muscle and its phrenic nerve were examined (5).

Ligation of salivary ducts. This was carried out on rats in ether anaesthesia. In a first series the parotid duct on one side and the ducts of the submaxillary and the sublingual glands on both sides were tied with a fine silk thread, the ducts of the latter two glands close to hilus causing at the same time an interruption of the parasympathetic nerve supply. In a second series both the parotid ducts and those of the submaxillary and sublingual glands on one side were ligated (5).

#### Drug treatments

6-hydroxydopamine. Under light ether anaesthesia 100 mg/kg of the drug as a chloride was injected into the tail vein of rats at weekly intervals (altogether 4 times). The drug causes a chemical sympathectomy; the adrenergic nerve fibres are selectively destroyed (see Thoenen and Tranzer, 1973). In order to maintain a chronic state of denervation a schedule recommended by de Champlain (1971) was followed. Salivary glands (9), and hearts and urinary bladders (10) were studied.

Hoechst 9980. This atropine-like substance (piperidino-ethyl-diphenyl-acetamide hydrochloride; Schaumann and Lindner, 1951) was given to rats s.c. in an amount of about 10 mg/kg body weight every 8 hr for 21 days (4).

Isoprenaline. Isoprenaline sulphate was given to rats in a



dose of 3 mg s.c. every 8 hr for 21 days; such a dose corresponded to about 15 mg/kg body weight (6).

#### Variations in the consistency of the food

Food, both the standard pelleted diet and the experimental diets were given ad libitum to rats; this was also the case for water. Liquid diet was prepared by mixing 2 parts of water with 1 part of a fine powder made from the standard diet (3). Dry bulk diet was made from the standard diet but the pellets contained 20 per cent cellulose instead of the usual 3 per cent (4). Compared with the rats given the usual standard pelleted diet, both the rats kept on the liquid diet and those kept on the dry, pelleted bulk diet tolerated their respective diet well as judged from their increase in body weights (3, 4).

#### Exercise

Rats were exercised in a motor driven rotating drum six to seven times weekly. The speed and running period were slowly increased. At the beginning of the 7th week the rats ran at 1500 m/hr for 1 hr/day. This training program was then continued. The hearts and salivary glands were examined (8).

#### Choline acetyltransferase activity

The method used to determine the activity of choline acetyltransferase was the one devised by Catherine Hebb (1963), described in detail by Nordenfelt (1963, 1965 a). In brief the method is as follows. Acetone dried powder is prepared from

the tissues to be studied. Choline acetyltransferase is extracted from the powder with cysteine-saline. The enzyme is then incubated in a system where only the concentration of the enzyme and not its substrates is rate limiting for the production of acetylcholine; this is achieved by using coupled systems. In the first reaction catalyzed by phosphate transacetylase (EC.2.3.1.8) acetyl-Coenzyme A is continuously formed from Coenzyme A and acetylphosphate. Acetylcholine is then synthesized in the second reaction. The acetylcholine formed is assayed on the eserinizied frog rectus muscle (see Feldberg, 1950; Nordenfelt, 1963).

Unless otherwise stated the total activity, i.e. the acetylcholine chloride formed per hour per organ or per pool of organs is considered in the following sections.

Experimental preparation and control preparation were incubated simultaneously and the choline acetyltransferase activities measured as a rule on the same frog rectus. The choline acetyltransferase activity is usually expressed in per cent of contralateral organ or of corresponding organ of litter mates. Comparisons between rats of the same litter and sex seem to be preferable; a recent report by Gaetani, Mengheri, Spadoni, Rossi and Toschi (1975) shows that the litter size affects the choline acetyltransferase activity and further, sexual differences in the choline acetyltransferase activity appear to exist, at least in certain brain structures of rats (Libertun, Timiras and Kragt, 1973; Luine, Khylichevskaya and McEwen, 1975).

## Statistics

Student's t-test for paired or unpaired samples was used. P values less than 0.05 were considered significant.

#### A. Parasympathetic denervation of salivary glands

When the auriculo-temporal nerve of the dog had been cut, about 30 per cent of the choline acetyltransferase activity remained in the parotid gland 1 week later (see Table). When both this nerve and the nerve fibres on the internal maxillary artery were cut the choline acetyltransferase activity was diminished to about 10 per cent. By including section of the facial nerve in the denervation procedure the enzyme activity was lowered even further; it reached in most cases values below the limit of detection (1). A similar pattern of reduction in cholinesterase-positive nerves of the gland was described by Garrett and Holmberg (1972) in a histochemical study; the largest amount of cholinesterase-positive nerves remained when only the auriculo-temporal nerve was cut and the smallest following the divisions of all the three nerves; even after the extended denervation procedure some cholinesterase staining nerve fibres were left, the source of which is unknown.

The small residual choline acetyltransferase activity found in the parotid glands of rats (4), cats and rabbits (Nordenfelt, 1963, 1964 c) after severance of the auriculo-temporal nerve can probably also be explained by the presence of unsevered cholinergic neurones, reaching the glands by channels outside the "classical" pathway, as is the case in the dog. In the cat support for this idea was given by Garrett (1966), who still could detect cholinesterase-positive nerves

in the gland after avulsion of the auriculo-temporal nerve and sympathectomy. Further, afferent stimulation of the glossopharyngeal nerve still caused secretion from the gland after section of the auriculo-temporal nerve (Ekström and Emmelin, 1974); in such denervated glands the choline acetyltransferase activity was reduced to the same level as that found by Nordenfelt (1963). Secretory cholinergic nerves were traced along the internal maxillary artery in this species, too (Ekström and Emmelin, 1974). In rats, also, the parotid seems to receive other cholinergic fibres than those in the auriculo-temporal nerve (Alm and Ekström, unpublished observation). When the latter nerve had been cut and degenerated, cholinesterase-positive nerves could still be found, and a cholinesterase inhibitor injected through the duct into the gland caused a small secretion, indicating that nerves were present, from the endings of which acetylcholine was leaking. Similar experiments indicate that also in rabbits the parotid gland receives cholinergic nerves outside the auriculo-temporal nerve (Alm and Ekström, unpublished observation). Nordenfelt (1964 c), suspecting that some postganglionic neurones were left after cutting the auriculo-temporal nerve, tried in vain to find any ganglion cells in the glandular tissue; he suggested tentatively that the residual choline acetyltransferase activity might be derived from the Schwann cells.

#### B. Parasympathetic decentralization of salivary glands

The choline acetyltransferase activity of the parotid gland of the dog was reduced to about 75 per cent (see Table) 1 week after section of the tympanic nerve (2). The secretory



response reflexly elicited by pouring citric acid on the tongue was totally or almost totally abolished by the decentralization, indicating that all or nearly all the preganglionic parasympathetic neurones had been cut. It should be pointed out that the section does not damage postganglionic cholinergic fibres, as judged by histochemical methods (Garrett and Holmberg, 1972). The percentage reduction of the enzyme activity was similar to that obtained in the cat parotid gland (Nordenfelt, 1964 b).

#### C. Effects of liquid diet

This regime is known to cause atrophy or reduction of the rate of growth of salivary glands (Hall and Schneyer, 1964; Wells and Peronace, 1967). In the present study parotid glands decreased in weight but not submaxillary and sublingual glands (3). The choline acetyltransferase activity was diminished in the parotid gland; this was seen already at an early stage. The enzyme activity of the other two major salivary glands was unchanged (Table). If expressed as a percentage the fall in the enzyme activity was of the same magnitude as that seen in decentralized parotid glands of dogs (2) and of cats (Nordenfelt, 1964 b).

This experiment shows that it is possible to induce a decrease in the choline acetyltransferase activity in postganglionic neurones without isolating the neurones surgically from the central nervous system.

#### D. Effects of dry bulk diet

A cellulose rich diet may increase the weight of salivary

glands (Wells and Peronace, 1967). Such a diet given for 3 weeks caused the parotid gland to increase, while the weights of the submaxillary and the sublingual glands were unchanged (4). The choline acetyltransferase activity rose in the parotid gland (Table) but not in the other glands. The two phenomena seen in the parotid gland were found to depend on an intact auriculo-temporal nerve, since they failed to appear when the nerve had been cut in advance.

#### E. Effects of exercise

After running sessions rats were found to spread saliva onto their body surfaces (8). A similar behaviour is observed when rats are in a hot environment; by evaporating saliva the rats cool themselves (Hainsworth and Stricker, 1970). After the period of physical training the salivary glands were weighed; all of them were lighter than their respective control glands. However, when related to body weights, the submaxillary gland weighed somewhat more, i.e. 6 per cent more than the controls (8). Increased submaxillary gland weight has previously been reported in trained rats by Donaldson (1931). Although the parasympathetic nerve fibres are of major importance for maintaining secretion in a hot environment (Stricker and Hainsworth, 1970; Elmér and Ohlin, 1971) no change in the choline acetyltransferase activity was found in the submaxillary or in the parotid glands. The increased secretory activity of the submaxillary glands, as judged from the increased gland weight to body weight ratio, was probably not sufficient to increase the activity of the acetylcholine synthesizing enzyme. For comparison it may be mentioned that the increase in enzyme

activity obtained in parotid glands after a cellulose rich diet was accompanied by a weight increase as large as about 45 per cent (4).

#### F. Effects of atropinization

This treatment led to an increase in the choline acetyltransferase activity in the parotid gland but not in the submaxillary gland (Table); the sublingual gland was not examined for its transferase activity (4). The increase in the enzyme activity was of the same percentage magnitude as that seen in the same gland after the regime of dry, cellulose rich diet. Whether the effects of the two kinds of treatments were additive could not be settled, since the rats exposed to the combined treatment reduced their food intake, as judged by body weight losses.

Although both the treatments, taken separately, have similar effects on the capacity to synthesize acetylcholine, it could not be expected that the weights of the salivary glands would be affected in the same way in the two instances, since the atropinization prevents the secretory impulses from reaching the gland cells. The treatment with the atropine-like drug caused, in fact, a weight reduction of both the submaxillary and the sublingual glands; a similar weight decrease of the submaxillary gland of the rat has previously been reported by Ohlin and Perec (1966) after treatment with Hoechst 9980. In contrast to these two glands, the parotid gland increased somewhat in weight. The gland enlargement was also seen after sympathetic denervation, excluding activity in sympathetic nerve fibres as the cause of this increase in

gland size; in such denervated glands the choline acetyltransferase activity also increased by about a similar percentage figure as was seen in the not denervated gland after the Hoechst 9980 treatment. The cause of the increase in parotid gland size after atropinization is very likely incomplete blockade of the transmitter action on the gland cells.

It is of interest to mention that treatment with pilocarpine, causing a profound salivary secretion (Schneyer and Hall, 1966), is followed by a decrease in the enzyme activity of the parotid gland (Ekström, unpublished observation).

#### G. Effects of duct ligation

When the function of some salivary glands is abolished by applying ligatures to their ducts both the weight (Wells and Peronace, 1964; Elmér and Ohlin, 1969) and the secretion (Elmér and Ohlin, 1969) of not duct-ligated glands are known to increase. After 4 weeks both the weight and the choline acetyltransferase activity of a parotid gland were increased in rats in which the ducts of the other large salivary glands had been tied; the increase in enzyme activity was about 30 per cent (5). When examined 3 and 7 days after the ligation procedure there was no change in the enzyme activity of the gland, although the size was increased markedly at the end of the week. The weight and the enzyme activity of the submaxillary and the sublingual glands were increased 4 weeks after tying the ducts of the two contralateral glands and both parotid glands; in the submaxillary gland the increase in enzyme activity as a percentage was the same as that found in the parotid gland, in the sublingual gland it was smaller (Table).

## H. Effects of isoprenaline

Prolonged treatment with this substance is known to cause gland enlargement and this is considered to depend on a direct action of the drug on the beta-adrenergic receptors of the gland cells (see Potho, 1966; Seifert, 1967; Emmelin, Schneyer and Schneyer, 1973). When rats were treated with isoprenaline over a period of some weeks the parotid gland increased 10-fold in size, the submaxillary 5-fold and the sublingual gland by 15 per cent (6). In spite of these effects on the gland size the choline acetyltransferase activity of the parotid and sublingual glands did not increase. It was considerably reduced in the submaxillary gland (Table); this reduction will be discussed later.

The increase in gland size observed is in agreement with a previous study by Schneyer (1962). It should be pointed out that the gain in weight cannot solely reflect increased secretory activity, since doses of isoprenaline below those evoking secretion are known to cause the parotid gland to increase in weight (Schneyer, 1969); and the dose of isoprenaline used in the present study evoked in acute experiments a very scanty secretion of saliva from the parotid gland, about a tenth of that from the submaxillary gland. It should also be mentioned that, although pilocarpine causes a more marked flow of saliva from the submaxillary gland of the rat than isoprenaline, the weight increase after pilocarpine is not at all as large as that seen after isoprenaline (Ohlin, 1966).

## I. Observations on the heart

Distribution of the enzyme. In cats, dogs, rabbits and rats an acetylcholine synthesizing capacity was demonstrated not only in the atria but in the ventricles also. In the ventricles this applied both to the basal and the apical part, the interventricular septum, and the outer walls of the chambers (7). The general concept has been that in mammals parasympathetic cardiac nerves supply the atria but not at all the ventricles, or at the most their basal parts. This view is, for instance, supported by early investigations showing choline acetyltransferase activity in the atria but not in the ventricles (Comline, 1946; Ebashi, 1954). However, particularly in recent years evidence has been produced demonstrating that the parasympathetic innervation of the ventricles is more extensive than assumed earlier; the literature was summarized by deGeest, Levy, Zieske and Lipman (1965) and by Higgins, Vatner and Braunwald (1973). One of the prerequisites for accepting the concept of a vagal (cholinergic) innervation of the mammalian ventricles is the demonstration of choline acetyltransferase activity; in this study using Hebb's method it was possible to show that the ventricles have an ability to form acetylcholine which is similar to, or even bigger than that of the atria (7, 8, 10).

Since the paper dealing with the distribution of choline acetyltransferase activity in the different parts of the heart (7) was published some other studies have appeared which demonstrate much higher values of enzyme activity than those of the present studies (Mahoney, Vogel, Salvenmoser and Boehme,



1971; White and Wu, 1973). However, White and Wu (1973) showed that the high values were explained by the use of radioactive methods analysing not only the  $^{14}\text{C}$ -labelled acetyl group in acetylcholine but also that in acetylcarnitine; when in their study endogenous carnitine was removed the acetylcholine forming capacity of the rabbit heart agreed with that of the present study using the method of bioassay. It should be pointed out that both carnitine and acetylcarnitine are considered to lack biological activity (Strack and Försterling, 1937; Thomitzek and Strack, 1964; Ryall, Stone and Watkins, 1964; see also Hebb and Morris, 1969). It appears likely that even the activity found after removal of endogenous carnitine can be higher than the true activity of choline acetyltransferase, since White and Wu (1973) demonstrate that the enzyme carnitine acetyltransferase present in hearts can under certain favourable conditions use choline as a substrate, thereby also synthesizing acetylcholine to some degree. It should be emphasized that the activity of carnitine acetyltransferase is extremely high in the heart; for comparison it may be mentioned that the activity of this enzyme in peripheral nervous tissue is about 200 times less than that of the heart (see Marquis and Fritz, 1965; McCaman, McCaman and Stafford, 1966); unfortunately parasympathetic postganglionic denervation of the heart cannot be carried out.

Effects of physical training. Cardiac hypertrophy is a common finding after training (Hatai, 1915; Hort, 1951; van Liere and Northup, 1957; Tipton, 1965). In this study both the atria and the ventricles increased in weight relative to the body weight

(S). The choline acetyltransferase activity was increased in the atria by about 20 per cent; it was not changed in the ventricles. These findings are in agreement with those of Herrlich, Raab and Giguee (1960), showing in the atria of trained rats increased, and in the ventricles unchanged concentrations of acetylcholine; as to the destruction of acetylcholine, cholinesterase activity appears to be unaffected by the training (Herrlich, Raab and Giguee, 1960; Tipton, Barnard and Tharp, 1966).

A low heart rate at rest after physical training is a phenomenon described in numerous investigations (see Tipton, 1965). Although the rate was not measured in this study, the training program was probably sufficient to cause a reduction in heart rate; for instance Watt, Foss and Block (1972) showed a decreased resting heart rate in rats after 8 weeks of running, the final 3 weeks at a speed of about 900 m/hr for 90 min daily. In the present study the rats were trained over a period of 11-14 weeks, the final 4-7 weeks at a speed of 1500 m/hr for 60 min.

The present finding of an increased capacity to synthesize acetylcholine in the atria after a period of physical training gives further support for the idea of an increased cholinergic activity as one of the mechanisms responsible for the low heart rate observed after training (Herrlich, Raab and Giguee, 1960; Tipton and Taylor, 1965; Tipton, Barnard and Tharp, 1966). The possibility of a decreased sympathetic nervous activity as the cause of the changed rate has been discussed by de Schryver and Mertens-Strythagen (1972). Since Herrlich, Raab and Giguee (1960) did not find any changes in

the concentrations of acetylcholine and cholinesterase in the ventricles, they explained the prolonged isometric tension period of the ventricles found after training (Mellerowicz, 1956; Klepzig, 1955; Raab, Silva, Marchet, Kimura and Star-cheska, 1960) as being due to a sympathetic inhibition.

#### J. Observations on phrenic nerve and diaphragm muscle

Section of the phrenic nerve on one side 4 weeks in advance led to an increase in the respiratory frequency of the rat by about 30 per cent (5). Although overuse of the innervated half of the muscle seems to occur, no weight increase was observed, a finding, which is in accordance with an observation by Sola and Martin (1953). In this innervated half of the muscle the choline acetyltransferase activity was increased by about 15 per cent. No change in the enzyme activity of the phrenic nerve could be found. Choline acetyltransferase activity has previously been demonstrated in the rat's phrenic nerve (Hebb, Krnjevič and Silver, 1964; Ekström and Emmelin, 1971) and in its diaphragm muscle where the enzyme has been shown to be localized in the neurones (Hebb, Krnjevič and Silver, 1964; Emmelin, Nordenfelt and Perec, 1966; Israël, 1970).

By the analysis of the enzyme activity not only in the muscle but in its phrenic nerve also, it was shown that the increase in the enzyme activity occurs particularly in the nerve endings. This preparation was chosen instead of the parotid gland and its auriculo-temporal nerve, a nerve which is difficult to study because of its early branching.

## K. Sympathetic denervation

Surgical denervation of salivary glands. In his studies, Nordenfelt (1964 a, 1965 b) found the choline acetyltransferase activity to be increased by about 20-30 per cent in the submaxillary and parotid glands of cats some weeks after excision of the superior cervical ganglion, whereas in the corresponding glands of rabbits no increase was found. It was also possible to demonstrate an increase (of about 40 per cent) in the rat's submaxillary gland. In the submaxillary gland of the dog, on the other hand, no change was observed. These findings seemed to suggest, as pointed out by Nordenfelt, that there are species differences, the increase occurring in the glands of some species but not in those of others. To test whether this interpretation is justified the observations were extended to some other glands. In the rat, no increase in the choline acetyltransferase activity could be demonstrated in the parotid or in the sublingual glands; in the submaxillary gland the percentage increase was about the same as that found by Nordenfelt (1964 a). In the dog, the parotid gland showed an increase of about 25 per cent. In the light of the present study it thus appears that in the same species one type of gland may show the effect of sympathectomy, whereas another type remains unchanged (Table).

When the submaxillary glands of some other species were examined it was found that the enzyme activity increased in the golden hamster and in the mouse by about 10-15 per cent; in the guinea-pig it did not change (9).

Since, of the salivary glands examined, the rat's sub-

maxillary gland showed the biggest increase in the enzyme activity, the development of the phenomenon was studied in this gland; an increase became obvious after about 2 weeks and thereafter the activity increased gradually. In the cat's submaxillary and parotid glands the increase in the activity was also noticed after about 2 weeks (Nordenfelt, 1965 b). Also the activity of the enzyme cholinesterase increases gradually in the submaxillary gland of the rat after sympathectomy (Nordenfelt, 1968).

Chemical denervation of salivary glands. Following this treatment the choline acetyltransferase activity was increased in the submaxillary gland of the rat by a similar percentage as that seen after surgical denervation, while it was unaffected in the sublingual gland (9). Thus a similar pattern as that observed after surgical sympathectomy is seen. It has been shown that a dose of 6-hydroxydopamine lower than that used in the present investigation causes disappearance of the adrenergic nerve terminals in the submaxillary gland of the rat (Goldman and Jacobowitz, 1971).

Chemical denervation of heart and urinary bladder. The use of 6-hydroxydopamine to cause a sympathetic denervation of the heart and the urinary bladder of the rat seemed suitable since extensive surgery is needed to achieve sympathetic denervation of the heart (Donald, 1974) and surgical interruption of the sympathetic nerve supply of the urinary bladder would also involve section of cholinergic neurones (Alm and Elmér, 1975). The substance is known to cause destruction of adrenergic nerve terminals in the rat's heart (de Champlain, 1971) and in

the rat's urinary bladder (Alm, personal communication).

Neither in the different parts of the heart, i.e. atria and ventricles, nor in the urinary bladder could a change in the choline acetyltransferase activity be shown (10). The dose and schedule of weekly administration of the substance were the same as those, which had effect on the enzyme activity of the submaxillary gland (9).



Choline acetyltransferase activity of salivary glands after various experimental procedures, expressed as a percentage of activity in control glands.

| Experimental procedure      | Species | Salivary glands  |                   |                  |
|-----------------------------|---------|------------------|-------------------|------------------|
|                             |         | Parotid          | Submaxillary      | Sublingual       |
| Aur.-temp. section          | Dog     | 29 <sup>1</sup>  |                   |                  |
|                             | Rat     | 3 <sup>3</sup>   |                   |                  |
| "Extended" parasymp.denerv. | Dog     | 1 <sup>1</sup>   |                   |                  |
| Parasymp. decentralization  | Dog     | 74 <sup>1</sup>  | -                 | -                |
| Liquid diet                 | Rat     | 72 <sup>2</sup>  | 98 <sup>2</sup>   | 96 <sup>2</sup>  |
| Dry bulk diet               | Rat     | 117 <sup>2</sup> | 101 <sup>2</sup>  | 102 <sup>2</sup> |
| Atropinization              | Rat     | 121 <sup>2</sup> | 100 <sup>2</sup>  | -                |
| Duct ligation               | Rat     | 132 <sup>2</sup> | 132 <sup>2</sup>  | 113 <sup>2</sup> |
| Isoprenaline                | Rat     | 99 <sup>2</sup>  | 63 <sup>2</sup>   | 99 <sup>2</sup>  |
| Sympathetic denervation     |         |                  |                   |                  |
| Surgical                    | Dog     | 124 <sup>1</sup> | 102 <sup>1+</sup> | -                |
|                             | Rat     | 100 <sup>1</sup> | 146 <sup>1</sup>  | 104 <sup>1</sup> |
| Chemical                    | Rat     | -                | 140 <sup>2</sup>  | 98 <sup>2</sup>  |

<sup>+</sup> figure taken from Nordenfelt (1964 a)

<sup>1</sup> compared with contralateral gland

<sup>2</sup> compared with corresponding gland of control litter mate

<sup>3</sup> compared with glands of controls

When it was originally suggested by Nordenfelt (1964 b) that the intensity of the traffic of impulses in a nervous pathway might, as a longterm effect, influence transmitter synthesizing enzymes at the nerve endings in an effector organ, this hypothesis was based on the finding that section of the preganglionic parasympathetic nerve reduced the choline acetyltransferase activity in salivary glands of cats and rabbits. This has now been found to apply to other species also and support for the hypothesis has been gained in various types of experiments which seem to have in common that for a long time period the flow of nerve impulses in a pathway is diminished or augmented from average conditions. Thus it is reasonable to suppose that administration of a liquid diet will greatly decrease the degree to which salivary glands are activated from the central nervous system. In parotid glands this was found to lead to a decline of the choline acetyltransferase activity about as large as if the preganglionic fibres had been cut. Conversely, an increase in the enzyme activity was recorded in situations where it could be assumed that the glands were stimulated above the ordinary because the diet was dry and bulky or dryness of the mucosa of the mouth and throat was produced by atropinization or duct ligation.

Turning to other organs, experiments on atria and diaphragms also showed that a rise in the activity of choline acetyltransferase occurred when it seemed very likely that these structures had for some time been exposed to an increased impulse stream in cholinergic nerves. The effect on

the choline acetyltransferase of disuse and overuse of striated muscle has also been the subject of some other recent investigations (Snyder, Riefenberick and Max, 1973; Riefenberick and Max, 1974; Diamond, Franklin and Milfay, 1974). There are, in addition, some further papers relating the degree of nervous stimulation to the choline acetyltransferase activity. When newborn rats were kept in darkness this was found to prevent the normal increment of the enzyme in visual structures of the central nervous system (Malètt and Timiras, 1968). An increased enzyme activity, on the other hand, was observed in the adrenals of aggressive mice (Goldberg and Welch, 1972) and in adrenals, sympathetic ganglia and brain of rats and chickens after injection of various drugs (Mandell and Morgan, 1970; Mandell and Knapp, 1971; Oesch, 1974).

In the case of adrenergic nerves, a relation between the intensity of the impulse traffic and the activity of enzymes engaged in the synthesis of the transmitter has also been considered. In sympathetically decentralized salivary and pineal glands Zieher and Pellegrino de Iraldi (1966) observed a reduced capacity to produce noradrenaline from l-Dopa which they attributed to a fall in the dopamine- $\beta$ -hydroxylase activity, and in decentralized sympathetic ganglia a fall in the tyrosine hydroxylase was demonstrated (Hendry, Iversen and Black, 1973). Particularly in recent years many investigations have been devoted to the effect on the catecholamine synthesizing enzymes of various situations involving stress or understimulation (see Molinoff and Axelrod, 1971; Pletscher, 1972; Thoenen, 1974).

The interesting phenomenon described by Nordenfelt

(1964 a, 1965 b) and further studied here, that sympathectomy induces an increase in the activity of choline acetyltransferase in some salivary glands may at first sight seem to fit into the general pattern as outlined above. It occurs only if the gland is connected with the central nervous system by way of the parasympathetic pathway. It is particularly marked in the submaxillary gland of the rat; this gland has a rich supply of sympathetic nerves, which have been shown to take part in digestive reflexes (Ohlin, 1968), and hence it could be thought that loss of these nerves might result in a compensatory increase in the impulse traffic in the parasympathetic nerves. The lack of rise in enzyme activity in organs such as the heart and the urinary bladder after chemical sympathectomy would also be understandable considering that here the two sets of autonomic nerves do not act in a similar but an antagonistic way. However, the view that loss of sympathetic impulses from the central nervous system is mainly responsible for the rise in enzyme activity noticed in some glands after sympathectomy becomes untenable in the light of the fact that no rise occurs when only the preganglionic sympathetic nerves are cut. This was found by Nordenfelt (1965 b) in experiments on cats and has also been seen in the submaxillary gland of the rat (Ekström, unpublished observation). The mechanism by which section of the postganglionic sympathetic neurone affects the level of the choline acetyltransferase in some glands is at present unknown. Presumably various factors may influence the enzyme. Hormonal effects have already been mentioned (page 8). Nordenfelt (1965 a, b) put forward the idea that some agent from the degenerating sympathetic nerves

might affect the postganglionic parasympathetic neurone, possibly inducing collateral regeneration. The present observation that intense stimulation of the  $\beta$ -adrenoceptors of the submaxillary gland of the rat by prolonged isoprenaline treatment lowers the activity of choline acetyltransferase may also be of interest in this context and, likewise, the report by Ohlin and Perec (1967) to the effect that repeated teeth amputations increase the activity of the enzyme in this gland. When searching for a mechanism responsible for the effect of sympathectomy on the enzyme in some glands, the following two facts ought to be taken into account. The effect is obtained particularly in glands in which the initial concentration of the enzyme is fairly low (see Table IV in paper 9). Further, it is larger the more intense the innervation with adrenergic fibres, which seems reasonable. These points were made by Nordenfelt (1964 a, 1965 a), and experiments of the present investigation, in which glands of many species were used, serve to emphasize them.

A striking feature in the present experiments was that it was the parotid gland which reacted with changes in choline acetyltransferase activity, and in weight, when the consistency of the food was altered or dryness of the oral and pharyngeal mucosa was produced. Obviously the submaxillary and sublingual glands were not unable to respond under these conditions but had less tendency to do so, as illustrated by the fact that their weight and enzyme activity did increase in the experiments where both parotid ducts (and the ducts of the contralateral submaxillary and sublingual glands) were tied. More than a century ago Claude Bernard (1856) pointed out that the

three pairs of large salivary glands have somewhat different functions. According to him the submaxillary gland is related particularly to taste, the sublingual to swallowing and the parotid to mastication. For instance, he called attention to the facts that the parotids are relatively much larger in horses than in dogs; in horses chewing is much more extensive than in dogs. In animals living in water the parotids are very small while the submaxillary glands are of the same size as in closely related terrestrial species. Claude Bernard also described experiments in which the consistency of the food given to a horse was varied and the volume of saliva secreted from the parotid gland was estimated: the secretion was much bigger when the food consisted of hay than when it was fresh bread. Semernina (1932, quoted by Babkin, 1950), working on dogs reached similar conclusions as Bernard: the function of the parotid gland is mainly to moisten and to cleanse the mouth cavity, thereby making mastication possible. In more recent years Dawes and Jenkins (1961, 1964) have shown the parotid and submaxillary glands of the human to respond unequally to different kinds of stimuli in the mouth. The parotid secreted twice as much saliva as the submaxillary gland on mechanical stimuli such as the presence of sand in the mouth and chewing wax or bread whereas strongly tasting materials stimulated both glands approximately equally.

The relative weight increase of the submaxillary gland in the rats exposed to exercise appears to be another example of selective activation of the glands, since it is this gland which is the primary thermoregulatory effector (Elmér and Ohlin, 1970, 1971; Hainsworth and Stricker, 1971; Rodland and



Hainsworth, 1973; Curé, 1973); the gland secretes and increases in size in a hot environment.

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